Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.3, No.12, 2013
www.iiste.org

Isolation and Identification by PCR and Analysis for Probiotic Properties of *Lactobacillus spp* from Dairy Products.

Anwar.A.Abdulla*, Mohammed.A.Jebor, Thikra. A. Abed, and Maha J. Noshee
Department of Biology-Biotechnology, College of Sciences, Babylon University, Iraq.
* E-mail of the corresponding author: alhussainybio@yahoo.com

Abstract
Lactic acid bacteria (LAB) are very significant to human health and due to their ability to produce some antibacterial substances and ability to inhibit pathogenic bacteria, they are commonly used as a natural food preservative to improve food safety and stability,. The present study was focused on isolation and characterization of *Lactobacillus spp* from dairy products at local markets of Babylon province of Iraq, by conventional and molecular methods using PCR. Additionally, the study and to demonstrate some of probiotic properties of these isolates. All isolates were phenotypically characterized including studying the microbiological, biochemical, effect of sodium chloride and pH during growth, carbohydrates test and characterizing the antimicrobial activity of *Lactobacillus spp* against pathogens. The present study demonstrates that *Lactobacillus spp* produced a bacteriocin- like inhibitory substance with a broad spectrum of antimicrobial activity directed against pathogenic indicator organism suggesting its protective value against enteric pathogens.
Keywords: *Lactobacillus*, identification, PCR, dairy product, antimicrobial activity, probiotic.

1. Introduction

Certain species of Lactobacilli are important and are gaining increasing attention in food fermentation industry because of their biotechnologically interesting properties (Reid *et al*.,2001).Based on their "Generally regarded as safe"(GRAS) status, Lactobacilli have been extensively studied for their molecular biology in order to improve their specific beneficial characteristics(Pouwels and Leer,1993).

The largest group of probiotic bacteria in the intestine is lactic acid bacteria (LAB). Probiotics are live microorganisms that are similar to beneficial microorganisms found in the human gut, and have emerged as a major balancing factor influencing gastrointestinal physiology and function (Diplock *et al*., 1999).

In food industry, LAB are widely used as starter cultures and have been recognized to be part of human microbiota (Holzapfel *et al*., 2001).In raw milk and dairy products such as cheeses, yoghurts and fermented milks, Lactobacilli are naturally present or added intentionally, for technological reasons or to generate a health benefit for the consumer, and therefore yoghurt is one of the best-known foods that contain probiotics (Oskar *et al*., 2004).

The genus *Lactobacillus* consists of a genetically and physiologically diverse group of Gram positive , rod shaped ,catalase negative , non-spore forming bacteria (MacFaddin,2000), Lactobacilli could be considered among the most important of all Lactic acid bacteria due to their role in various food and feed fermentations and production of several important metabolites. Additionally, they play important role in the prevention of food spoilage, intoxication and infection by acting as antagonists against other pathogens through the production of antimicrobials and bacteriocine (Holzapfel *et al*., 2001; Hirano *et al*., 2003).

The development of a molecular culture- independent detection methods such as PCR is a simple technique that quickly amplifies specific sequences of target DNA from indicator organisms appears to be invaluable in the case of probiotics particularly *Lactobacillus spp*.(Roy *et al* ., 2000 ;Ventura and Zink , 2002).

Nucleotide base- sequences16S ribosomal DNA (rDNA) of *Lactobacillus spp*. provides accurate basis for phylogenetic identification and analysis (Tabataba*ce et al*., 2005).

The aim of this study was to use PCR for the detection of strains of *Lactobacillus spp*. Isolated from dairy products and also to demonstrate some of probiotic properties of these isolates.

2. Materials and Methods

Collection of samples: Eighty eight samples of dairy products including raw milk, cheese, yoghurt, and cream were collected from local markets of Babylon province of Iraq due to their wide acceptance among the consumers. Immediately after collection, the samples were stored in sterile containers at 4C.

Media and samples preparation: growth media used in this study were MRS broth (Himedia, India), MRS agar (Himedia, India), nutrient agar (Oxoid, England). Media were prepared according to manufacturer's instructions. All media and instruments were autoclaved for 15 min at 121˚C before use. One gm. of each sample was separately suspended in 100 ml of MRS broth of pH 6.5 and homogenized. A fivefold dilutions were made from each homogenized sample and all dilutions were incubated for 24 hours at 37˚C under anaerobic condition in the presence of 5 % CO2. A loopful of each culture was streaked on to the MRS agar plate and plates were

incubated under anaerobic condition at 37˚C for 24 hours (Sneath *et al*., 2009). Finally, a single colony of *Lactobacillus* was isolated based on their colony morphology and specific biochemical tests (Gram staining, oxidase, catalase, motility test, starch hydrolysis, growth at 10°C and 45°C in MRS broth, growth in (4, 6, 8, and 10percentNaCl as a tolerance test. Fermentation of carbohydrates were determined as described by Sneath *et al*., (2009) including glucose, sucrose, fructose, galactose, maltose, mannonse, and lactose.

Genetic- identification

- **Genomic DNA preparation**

DNA was extracted from the isolates by the wizard genomic DAN purification kit (Promega/USA) according to the manufacturer's instructions with several modifications. Purity and concentration of DNA were measured by using Nano Drop-spectrophotometer. Results of DNA purity and concentration (ng/µl) were recorded and plotted automatically. The Nano Drop-spectrophotometer measures DNA purity and concentration according to the following equations:

DNA purity = Abs. at 260nm / Abs. at 280nm

The purity of DNA ranged between1.7 to 1.9, and could be safely stored at - 20 °C until used.

- **PCR genes-specific for Lactobacilli and Gel Electrophoresis**

The specific primers were synthesized at AccuOligo / Bioneer/Korea).

Synthesized primers were provided in a lyophilized form, which were re-dissolved in TE buffer (pH 8) to a final concentration of 100 pmoles/µl, and stored at -20°C. The sequence of the primer set Lacto -16S forward - was **5'…..GGA ATC TTC CAC AAT GGA CG…..3'** and the primer set Lacto -16S reverse - was **5'…..CGC TTT ACG CCC AAT AAA TCC GG …..3'** Amplifications were carried out in 30 µl volumes containing (10 pmole /µl) of each primer, 2x Taq PCR Pre – Mix (SolGent™ 2x Taq PCR Pre – Mix , SolGent Co.,Ltd.) , and 200 ng genomic DNA. Amplification was achieved in 40 cycles using a GTC thermal cycler (Cleaver Scientific, UK).Prior to the first cycle, DNA was denatured at 95ċ for 3 min .Subsequently, each cycle consisted of denaturation at 95 ˚C for 30 sec., followed by annealing at 61 ˚C for 40 ˚C. Elongation was carried out at 72 ˚C and the extension time at 1 min. Subsequently, a final elongation was performed at 72˚C for 5 min., and the holding temperature was 10 sec. The PCR profiles were visualized after staining with ethidium bromide under ultraviolet light. A DNA molecular weight marker (SolGent Co., Ltd,Korea) was used to measure the weight of the fragments.

Determination of optimal growth:.

For the determination of pH for optimum growth of the isolates, 100µl overnight culture of the isolates was inoculated into MRS broth with varying pH ranging from (3-8). The pH was adjusted with concentrated Hcl or NaOH .The inoculated broths were incubated under anaerobic condition for 24 h at 37˚C in the presence of 5% CO2. Bacteria growth was measured using a spectrophotometer at 560 nm (Hoque *et al*., 2010).

Measurement of NaCl tolerance:

For the determination of Nacl tolerance, all isolates were grown in MRS broth supplemented with different concentrations of Nacl (4, 6, 8, and 10percent) that were inoculated after sterilization with 1% (v/v) of overnight culture of *Lactobacillus* and then were incubated an aerobically for 24h at 37˚C.The bacterial densities were determined by visual measurement of their turbidity and were classified as Maximum growth (++), normal growth (+), and no growth (-) (Hoque *et al* .,2010).

Antimicrobial activity against indicator organisms:

Antimicrobial effects of *Lactobacillus spp* against (*Serratia spp, Vibrio spp, Enterococcus faecalius, Morganella morganii, Staphylococcus aureus,* and *Salmonella typhi*) were determined by the agar diffusion method. Overnight cultures of the indicator strains were used to inoculate agar growth media (BHI Himedia, India) plates that were incubated at 37˚C. Wells of 5mm diameter were cut into the plates. To detect antibacterial activity of *Lactobacillus spp*. 10 ml of broth was inoculated with each strain of *Lactobacillus spp*., and was incubated at 37˚C for 48 h, cell free – solution was obtained by centrifuging the culture (6000 x g for 15 min), and then were followed by filtration of the supernatant through a 0.2 µm pore size, the filtered supernatant were neutralized by 1N NaOH to pH 6.5, then 50µl of supernatant fluid was added to each well and incubated at 37˚C for 24 h followed by measurement of growth inhibition zones (Topisirovic *et al*.,2006).

3. Results

Isolation and Identification: Ten *Lactobacillus*-suspect bacterial isolates were obtained from 88 samples of dairy products (raw milk, cheese, yoghurt and cream).The isolates were Gram positive, rod-shaped, oxidase

negative, catalase negative, non-motile, indol- negative and starch hydrolysis negative. Their characteristics are listed in table 1.The carbohydrates fermentation patterns of the isolates are listed in Table 1.

Molecular identification by PCR:

Primary affiliation based on the biochemical results facilitated the choice of appropriate molecular methods for further genes identification. All bacterial isolates in this study were tested by PCR genes – specific for identification of *Lactobacillus spp*. While the conventional identification methods revealed that there were 10 isolates belonged to *Lactobacillus spp*,, only 6 isolates were identified to belong to *Lactobacillus spp* based on the PCR method. (Fig 1).

pH and optimal growth:: The influence of pH was tested in a range of highly acidic (**pH** 3) to neutral (**pH** 7) and alkaline (**pH** 8). We have observed the maximum growth, by measurement of bacterial densities of Lac 1 isolated from raw milk to be at pH 5.0 and maximum growth of Lac 6 isolated from cheese at pH 6. The results of experiment are shown in Fig (2). There is a strong correlation between the pH and the growth of the Lactobacilli, the maximum growth was enhanced when the culture was controlled at pH 5.0 and 6.0.Survival could also be observed that at acidic pH values of 3.0 and 4.0.

Influence of various Nacl concentrations:

Isolated Lactobacilli were able to tolerate growth 4, 6, 8, and 10 percent of Nacl in MRS broth.. However, bacterial growth was correlated with various Nacl concentrations in the media with optimal growth being optimal at 4 percent Nacl while concentration of 10% Nacl significantly inhibited the growth of Lactobacilli with exception of Lac 1 that could grow at this Nacl concentration. Results of the experiment are shown in Table 2.

Antimicrobial activity: Isolates of pure local cultures for *Serratia spp, Vibrio spp, Enterococcus faecalius, Morganella morganii, Staphylococcus aureus, and Salmonella typhi*, were kindly provided by Hilla hospital microbiology lab. Babylon, Iraq, and their identifications were confirmed according to Holt *et al*., (1994).Antibacterial activity of cell – free supernatant was evaluated on the provided clinical strains of *Serratia spp, Vibrio spp, Enterococcus faecalius, Morganella morganii, Staphylococcus aureus, and Salmonella typhi* using agar well diffusion method (Topisirovic *et al*., 2006). The results of the experiment showed that the *Serratia spp* is the most affected by filtered supernatant of the six *Lactobacillus spp* among the clinical isolates while the *Vibrio spp* and *E.faecalius* were least affected, whereas the rest of the isolates showed varying response towards the filtered supernatant, Fig(3). The results of present study, showed that Lac 1 have a broad spectrum of activity against the tested clinical bacterial isolates.

4. Discussion

In the present study, all physiological and biochemical characteristics of the Lactobacilli isolated from the dairy products were identical to those reported by Cullimore (2000).

Characterization of ten selected isolates was initially conducted by cell morphologyand by physiological and biochemical tests. Results of these tests and their limits are shown in Table 1. It is difficult to identify a microorganism only by using the changes in pH as in indicator of growth in the presence of different sugars because of the various cut-off points used to determine appositive or a negative reaction (Fitzsimons *et al*., 1999).Furthermore, results from -specific PCR analysis revealed that only six of 10 *Lactobacillus*-suspect isolates belonged to *Lactobacillus spp*.

The majority of the LAB possesses an inducible acid tolerance response (ATR) which is also known as the acid adaptive response. This property improves the survival of adapted cells upon exposure to lethal acid challenge (Cotter and Hill, 2003). pH is an important factor which can dramatically affect bacterial growth. In the present study, we assessed the growth of *Lactobacillis spp* in various ranges of pH (3 -8), to determine the pH for optimum growth. In this study, it was found that the Lac 1, isolated from milk, had a maximum growth (OD =2.630) at pH 5.0 that exceeded the growth of other Lactobacilli which had their optimal growth at different pHs, Fig (2).

Nacl is an inhibitory substance which may inhibit growth of certain types of bacteria. Growth at different Nacl concentrations was observed; all of the isolates have the ability to grow at 4, 6, and 8 percent concentrations of Nacl. Growth of all isolates was inhibited at 10 % Nacl concentration, however Lac1 could grow at this concentration. Our experimental results are in agreement with the findings of Elezete and Carlos (2005), in case of Lactobacilli isolated from gastrointestinal tract of swine that were tolerable to 4-8 % Nacl.

The growth-inhibiting activity of LAB to other bacterial spp has generally been attributed to the fact that *Lactobacillus spp* lower the pH and / or produce lactic acid,for example strains of *L. acidophilus* ,*L. casei* subsp,*rhammnosus* and *L. bulgaricus* inbibited the growth of clinical isolates of *H.pylori* (Midolo et al., 1995), while *L. casei subsp rhammnosus* strain Lcr 35 reduced the growth of enteropathogenic *E. coli* and *Klebsiella*

pneumonae (Forestier *et al*., 2001).The data reported by Fayol-Messaoudi *et al*., (2005) showed that the strains induce complete inhibition of the growth *Salmonella spp* that results mainly from the effect of an acidic pH .
Antimicrobial activity of *Lactobacillus* strains against bacterial pathogens was revealed to be multifactorial and to include the production of hydrogen peroxide , lactic acid, bacteriocin-like molecules and unknown heat – stable , non-lactic acid molecules (Servin,2004;Olanrewaju,2007).

5. Conclusion

Results of this study suggest that only a few Lactobacillus isolate may possess unique characteristics that worth's further investigation for the identification of the mechanism of of it antimicrobial effect against specific pathogens as well as its ability for optimal growth and survival in the presence of high concentrations of Nacl in their growth medium. We conclude that Lactic acid bacteria (LAB) from fermented products may ac t as a reservoir of antimicrobial-resistance genes (Florez et al., 2005). These bacteria could act as bio-therapeutic microorganisms and might be good candidates to overcome the growing challenge of nosocomial infections due to multi-drug resistant strains.

References:

Cotter,P.D. and Ltill,C.(2003).Mol.Biol.Rev.67:429-453.

Cullimore, D.R., 2000. Practical Atlas for Bacterial Identification. CRC/Lewis Publishers, London, ISBN: 9781566703925, Pages: 209.

Diplock,A.T.;Aggett,P.;Ashwell,M.;Bornet,F.;Fern,E. and Roberfroid,M.(1999).Scientific concepts of functional food sci.in Europe:consensus document.Brit.J.Nutr.Suppl.1:1-28.

Elizeter,D.F.R.P. and Carlos,R.S.(2005).Biochemical characterization and identification of probiotic *Lactobacillus* for swine. B.CEPPA, Curitiba, 23:299-310.

Fayol-Messaoudi,D.;Berger,C.N.;Connier-Polter,M.H.;Lievin-Le Moal,V.and Serviu,A.L.(2005).pH-lactic acid and non-lactic acid-dependent activities of probiotic Lactobacilli against *Salmonella enterica* serovar Typhimurium.Appl.Environ.Microbiol.71:6008-6013.

Fitzsimons, N.; Cogan, T.; Condon, S. and Beresford. (1999).Phenotypic and genotypic characterization of non-starter lactic acid bacteria in mature cheddar cheese.Appl.Environ.Microbiol.65:3418-3426.

Florez, B.A.;Delgado,S. and Mayo,B.(2005).Antimicrobiol susceptibility of Lactic acid bacteria isolated from a cheese environment can.J.Microbiol.51:51-58.

Forestier,C.;De Champs,C.;Vatoux,C. and Joly,B.(2001).Probiotic activities of *Lactobacillus casei, rhammosus* :*in vitro* adherence to intestinal cells and antimicrobial properties.Res.Microbiol.152:167-173.

Hirano J, Yoshida T, Sugiyama T, Koide N, Mori I, Yokochi T (2003). The effect of *Lactobacillus rhamnosus* on enterohemorrhagic *Escherichia coli* infection of human intestinal cells *in vitro*. Microbiol. Immunol., 47: 405-409.

Holt, J. G., Krieg, N.R., Sneath, H.A., Staley, J.T. and Williams, S. T. (1994). Berge's manual of determinative Bacteriology (9th ed). Williams and Wilkins.

Holzapfel, W.H.; Haberer, P.; Geisen, R.J.; Bjorkroth and Schillinger,M.(2001). Taxonomy and important features of probiotic microorganisms in food nutrition.Am.J.Clin.Nutr.73:365-373.

Hoque,M.Z.;Akter,F.;Hossain,K.M.;Rahman,M.S.M.;Billah,M.M. and Islam ,K.M.D.(2010). Isolation, Identification and Analysis of Probiotic Properties of *Lactobacillus Spp.* From Selective Regional Yoghurts. World Journal of Dairy & Food Sciences 5 (1): 39-46.

MacFaddin, J.F. (2000). Biochemical Tests for Identification of Medical Bacteria. 3rd ed. Lippincott Williams and Wilkins, USA.

Midolo,P.D.;Lambert,J.R.;Hull,R.;Luo,F.and Grayson,M.L.(1995).In vitro inhibition of *Helicobacter pylori* NCTC 11637 by organic.

Olanrewaju, O. (2007).Antagonistic effect of *Lactobacillus* isolates from kunnu and cow milk on selected pathogenic microorganisms. Internet J. Food safety.vol.9:63-66.

Oskar, A.; Meydani,S.N. and Russell,R.M.(2004).Yogurt and gut function.Am.J.Clin.Nutr.80:245-56.

Pouwels,P.H.and Leer,R.J.(1993).Genetics Lactobacilli:plasmids and gene expression,Antonine Van Leeuwenbock.64:85-107.

Reid,G.;Beuerman,D.;Heinemanne and Bruce,AW.(2001).FEMS Immunal.med.microbiol.32:37-41.

Roy D, Ward P, Vincent D, Mondou F (2000). Molecular identification of potentially probiotic lactobacilli. Curr. Microbiol. 40: 40-46.

Servin,A.L.(2004).Antagonistic activities of Lactobacilli and Biofidobacterium against microbial pathogens.FEMS microbial.Rev. 28:405-440.

Sneath, P.H.S.; Mair, N.S.;Sharpe,M.E.and Holt,J.C.(2009).Berger's manual of systematic bacteriology.Baltimore,Williams and Wikins.
Tabatabaee, A.; Assadi, M.M.;Noohi,A.and Sajadian,V.(2005).Isolation of biosurfactant producing bacteria from oil reservoirs.IJEHSE.2(1).
Topisirovic,L.;Kojic,M.;Fira,D.;Golic,N.;Strahinic,I. and Lozo,J.(2006).Potential of lactic acid bacteria isolated from specific natural riches food production and preservation. International journal of Food Microbiology.112 (31):230-235.
Ventura, M., and R. Zink**.** 2002. Specific identification and molecular typin analysis of Lactobacillus johnsonii by using PCR-based methods and pulsed-field gel electrophoresis. FEMS Microbiol. Lett. 217**:**141-154.

Table 1. Biochemical test results of *Lactobacillus* spp.

Morphological &biochemical	**Lac 1**	**Lac 2**	**Lac 3**	**Lac 4**	**Lac 5**	**Lac 6**	**Lac 7**	**Lac 8**	**Lac 9**	**Lac 10**
Gram stain	+	+	+	+	+	+	+	+	+	+
Oxidase test	-	-	-	-	-	-	-	-	-	-
Catalase test	-	-	-	-	-	-	-	-	-	-
Motility test	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	+	-	+	-	+	-	-	-	-	-
Indol test	-	-	-	-	-	-	-	-	-	-
Growth at 10 ˚C	+	–	–	–	±	±	–	–	–	–
Growth at 45 ˚C	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	±	+	±	±	+	–	+	+
sucrose	±	–	–	±	–	+	–	±	–	+
fructose	+	–	–	–	–	–	–	–	–	+
galactose	–	–	+	–	–	+	+	+	–	–
maltose	+	–	–	–	+	–	–	–	–	–
mannonse	+	–	–	–	+	+	+	–	±	+
lactose	±	±	–	±	–	±	±	–	±	–

(+): positive result; (-): negative result; (±): variable result.

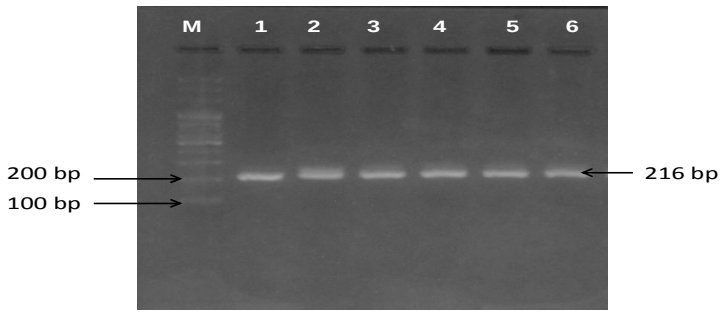

Figure 1. Amplified PCR products from *Lactobacillus spp* with primer set Lacto- 16S-F + Lacto16S-R.Lane (1-6) PCR products amplified from 6 *Lactobacillus spp*. Lane M: 100 bp markers.

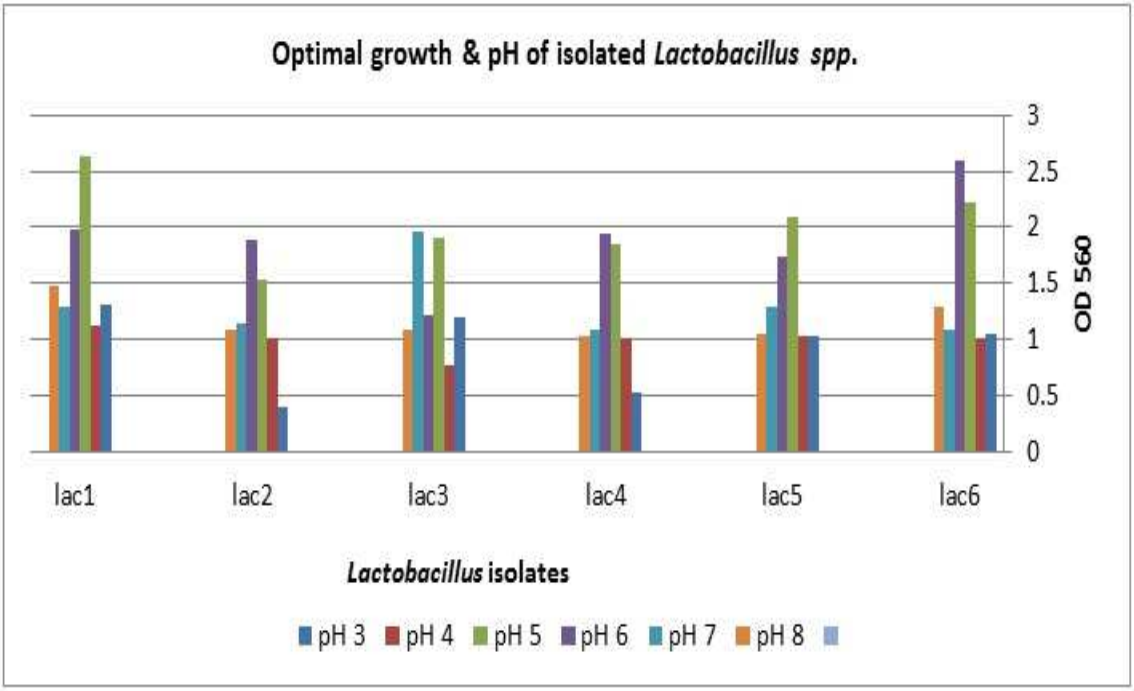

Figure 2.Optimal growth and pH of isolated *Lactobacillus spp*

Table 2. Tolerance to Nacl results of *Lactobacillus* spp.

Con..of Nacl No. % of isolates	4%	6%	8%	10%
Lac 1	++	++	+	+
Lac 2	+	+	+	-
Lac 3	++	+	+	-
Lac 4	++	+	+	-
Lac 5	++	+	+	-
Lac 6	++	+	+	-

(++): Maximum growth; (+): normal growth ;(-): no growth.

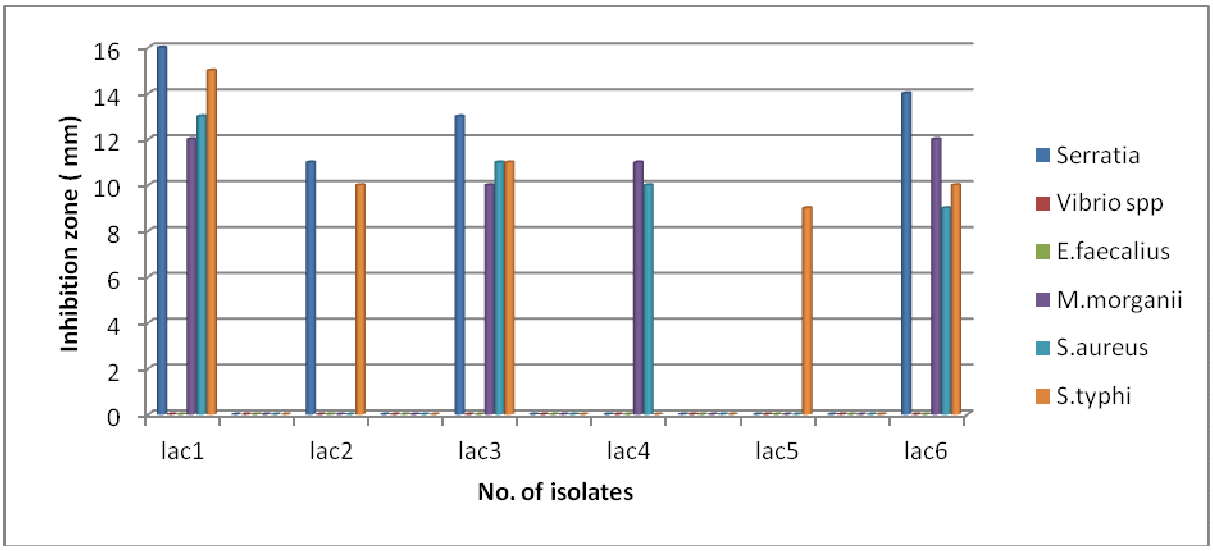

Figure 3.Antimicrobial activity of *Lactobacillus spp* against 6 indicator bacterial spp.